Title: Physiological, but not fitness, effects of two interacting haemoparasitic infections in a wild rodent

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Abstract

In contrast to the conditions in most laboratory studies, wild animals are routinely challenged by multiple infections at once, and these infections can interact in complex ways. This means that the impact of a parasite on its host's physiology and fitness cannot be fully assessed in isolation, and requires consideration of the interactions with other co-infections. Here we examine the impact of two common blood parasites in the field vole (*Microtus agrestis*): *Babesia microti* and *Bartonella* spp., both of which have zoonotic potential. We collected longitudinal and cross-sectional data from four populations of individually-tagged wild field voles. This included data on biometrics, life history, ectoparasite counts, presence/absence of microparasites, immune markers and, for a subset of voles, more detailed physiological and immunological measurements. This allowed us to monitor infections over time and to estimate components of survival and fecundity. We confirm, as reported previously, that *B. microti* has a preventative effect on infection by *Bartonella* spp., but that the reverse is not true. We observe gross splenomegaly following *B. microti* infection, and an increase in IL-10 production along with some weight loss following *Bartonella* spp. infection. However, these animals appeared otherwise healthy and we detect no impact of infection on survival or fecundity due to the two haemoparasite taxa. This is particularly remarkable in the case of *B. microti* which induces apparently drastic long-term changes to spleen sizes, but without major adverse effects. Our work sheds light on the ecologies of these important zoonotic agents, and more generally on the influence that interactions among multiple parasites have on their hosts in the wild.

Key words: disease ecology; co-infection; immunology; *Babesia microti*; *Bartonella*; *Microtus agrestis*

1. Introduction

An individual animal in its natural environment is likely to harbour multiple parasitic infections (Petney and Andrews 1998, Cox 2001). These parasites can interact with one another in complex ways, meaning that even low-virulence infections can indirectly influence the host's physiology and fitness by changing the course of more virulent infections (Randall, Cable et al. 2013). Understanding how parasites interact in a natural setting provides a vital complement to more controlled, lab-based studies (Pedersen and Babayan 2011), and in particular it allows us to study parasites in the context of the natural co-infections which may occur.

Two parasites may interact with one another in several distinct ways. Commonly, one parasite may adversely affect the host's condition, or cause a shift in immunity, that then makes it easier for another to invade, or to increase in abundance (Cox 2001). Alternatively, one infection can make it harder for a second to become established (van Duivenvoorde , Voorberg-van der Wel et al. 2010, Randall, Cable et al. 2013), for example through direct competition for resources (Johnson and Buller 2011), cross-reactivity of antibodies (Naus, Jones et al. 2003), or a shift in host cytokine production (Graham, Cattadori et al. 2007). These interactions can give counter-intuitive outcomes for the host: one type of infection (particularly if it has low virulence) may have a net positive effect on host fitness if it reduces the abundance or prevalence of a second pathogen (Randall, Cable et al. 2013, Wuerthner, Hua et al. 2017). These indirect effects are important when considering treatment or removal of a parasite during disease management, and species or strains of pathogen should not be considered in isolation. It is vital to understand any changes in susceptibility to other parasites due to the intervention that might lead to unintended negative consequences (Graham 2008, Fenton 2013, Johnson, de Roode et al. 2015).

In wild field voles, *Microtus agrestis*, a negative interaction has been described between two common taxa of blood parasites *Babesia microti* and *Bartonella* spp. (Telfer, Lambin et al. 2010, Sherlock, Xifara et al. 2013). The protozoan *B. microti* infects a number of rodent species, often without obvious symptoms. It is transmitted by ixodid ticks, which can also spread the infection to humans, causing babesiosis (Telford, Gorenflot et al. 1993, Homer, Aguilar-Delfin et al. 2000). The course of an infection varies, but a typical

sequence in rodents consists of acute and chronic phases. The acute phase lasts several weeks, during which time *B. microti* infects the red blood cells (RBCs) of the host and may cause severe anaemia. Between 20 and 50 % of the RBCs can be infected, the packed cell volume (PCV) may be reduced to as little as 20 %, and the spleen becomes greatly enlarged (Van Peenen and Healy 1970, Cullen and Levine 1987, Watkins, Moshier et al. 1991). Mortality in otherwise healthy animals is low, however, and after 3-4 weeks most have little or no evidence of infection in the blood. Nonetheless, previously infected animals retain a chronic carrier status (Lykins, Ristic et al. 1975, Homer, Aguilar-Delfin et al. 2000) and *B. microti* remains detectable by PCR indefinitely (Bown, Lambin et al. 2008).

Bartonella is a genus of gram-negative bacteria that infects a wide range of mammalian hosts (Breitschwerdt and Kordick 2000). It is transmitted by blood-sucking arthropods, primarily fleas (Bown, Bennett et al. 2004, Gutiérrez, Krasnov et al. 2015), and invades the RBCs of the host (Breitschwerdt and Kordick 2000). Like *B. microti,* some *Bartonella* spp. can be transmitted to humans via arthropod vectors, where they cause several different diseases including bartonellosis and cat scratch disease (Anderson and Neuman 1997, Oksi, Rantala et al. 2013, Vayssier-Taussat, Moutailler et al. 2016).

In field voles, *Bartonella* spp. (henceforth "*Bartonella*") and *B. microti* show a negative interaction, with few animals showing coinfection despite relatively high prevalence of the individual infections (Telfer, Lambin et al. 2010). This interaction appears to be unidirectional; that is, *B. microti* reduces the chance of infection with *Bartonella*, but the reverse is not the case (Sherlock, Xifara et al. 2013). Particularly in light of the zoonotic potential of the two infections, it is valuable to study them in combination to understand the fitness effects on the host, and to learn more about the dynamics of their interaction.

Here, we aim to explore the consequences of *Bartonella* and *B. microti* infections for wild-caught field voles. We make use of longitudinal data from multiple captures of the same individuals to examine infection sequences and changes in immunology over time to help separate cause from effect. We also use more detailed physiological and immunological data taken from destructive cross-sectional sampling. We confirm the findings of Sherlock, Xifara et al. (2013) that *B. microti* reduces susceptibility to *Bartonella*, but not the reverse. We find major physiological changes in the case of *B. microti* infection (characterised by

splenomegaly) but, surprisingly, no evidence of adverse fitness consequences. Finally, we are unable to find any support for the hypothesis that *B. microti* infection has any indirect, positive effect on the host via its negative interaction with *Bartonella*.

2. Materials and Methods

This paper uses data that have been previously analysed in Jackson, Begon et al. (2011) and Jackson, Hall et al. (2014). Here we give a short summary of the data collection methods; for more details see the two references above. We carried out all procedures under UK Home Office licence regulations.

2.1. Fieldwork

Wild field voles were trapped at four different sites in Kielder Forest, Northumbria, UK: two sites in 2008-2009 and a further two in 2009-2010. At each site, 150 Ugglan small mammal traps were laid out in a grid spaced approximately 3-5 m apart. During monthly trapping sessions, traps were checked five times over the course of 3 days, and newly trapped field voles were injected with a Passive Integrated Transponder (PIT) tag for unique identification. This approach allowed us to build up a longitudinal record for voles that were caught across multiple sessions. On capture, we recorded sex and body mass of the voles, as well as reproductive status (males were considered to be reproductively active if they had descended testes; females if they were pregnant or had perforate vaginas). We also conducted a thorough visual inspection of the fur to count ectoparasites including ticks and fleas, and took a drop of blood from the tail into 500 µl RNAlater, for use in pathogen detection and immune assays (see 2.3 and 2.4 below).

2.2. Cross-sectional data

At each trapping session we also retained a small number of individuals (up to 20) that were transported to the laboratory. These individuals were killed and dissected in order to collect more detailed, invasive measurements. For this component of the study, we focused on male voles only: we did not wish to remove pregnant females from the wild population, and data collected from non-pregnant females would therefore represent a biased sample.

Voles were killed by an overdose of chloroform, following which they were immediately weighed and then exsanguinated. The resulting blood samples were divided into two aliquots: one was used in pathogen detection (see 2.3), and the other for estimation of PCV. The latter blood sample was spun for 3 minutes in a microhaematocrit centrifuge (Hawksley) to separate cells from plasma and the ratio of the two (PCV) was calculated. We measured body length from snout to vent (SVL), removed the spleen and measured its wet mass before setting up splenocyte cultures (see 2.5). We conducted a thorough search of the fur for ectoparasites.

2.3. Pathogen detection

We extracted DNA from blood samples and amplified pathogen-specific sequences using PCR. See Bown, Lambin et al. (2008) for details of *B. microti* detection and Telfer, Bown et al. (2005) for *Bartonella*. For longitudinal samples, we used an aliquot of the tail blood samples and extracted DNA using isopropyl alcohol precipitation (see Jackson, Hall et al. 2014 for details). For cross-sectional samples in 2008, we used blood collected on filter paper and DNA extraction using Tris-EDTA buffer, as described in Bereczky, Mårtensson et al. (2005). As this method appeared to have a lower sensitivity than expected, in 2009 we switched to extracting from pelleted RBCs after centrifugation, using alkaline digestion as described in Bown, Begon et al. (2003).

2.4. Blood immunology

We used two-step reverse transcription quantitative PCR to estimate the expression levels of the genes Interferon gamma (IFNγ), Gata3 and Interleukin 10 (IL-10) in peripheral blood samples. Having only a limited volume of blood from each individual, we selected these three genes as broadly representative of Th1 (IFNγ), Th2 (Gata3) and regulatory/anti-inflammatory (IL-10) immune responses. Samples were run in duplicate, and each 96-well plate included no-template controls. Expression was standardised against Ywhaz and Sdha as endogenous control genes, which were identified by geNorm analysis (Vandesompele, De Preter et al. 2002) as the most stably expressed in the blood samples from a panel of seven candidate house-keeping genes. Expression values were normalised relative to a calibrator sample, using the ΔΔCT method (Livak and Schmittgen 2001).

2.5. Splenocyte cultures

After disaggregating spleen cells and lysing RBCs, we incubated the splenocytes (at a concentration of 2 x 10^6 cells per ml, total volume 300 µl) for 96 h in two assays: one stimulated with the mitogen phytohaemagglutinin (40 µg per ml), and the other an unstimulated control. Assays for each individual and condition were replicated four times. After the incubation period, we estimated gene expression levels using two-step reverse transcription quantitative PCR, amplifying the sequences for IFNγ, Interleukin 2 (IL-2) and T-box transcription factor TBX21 (T-bet) as markers for the Th1 immune response. Ywhaz was selected as the endogenous control gene in this case, being the most stably expressed in the splenocytes out of seven candidate house-keeping genes. Detailed methodology can be found in Jackson, Begon et al. (2011).

2.6. Statistical analysis

Prior to statistical modelling, we transformed (natural log + 1) gene expression variables and quantitative parasite measures, and removed any rows containing missing values relevant to the model in question. Because different models used different sets of variables, this led to varying sample sizes (see Table 1).

All models included fixed effects of season and site (with "year" also implicitly accounted for, as site is nested within year), and for reproductive status and sex in cases where both factor levels were present. Further predictors are listed separately under individual models and in Table 1. We modelled season as a sinusoid curve with a period of one year, using one sine and one cosine curve combined, following previous evidence that several infections show periodic variation in prevalence over the course of the year (Telfer, Lambin et al. 2010). During model selection, these two curves were treated as a single unit.

We calculated an index of body condition as the residual values from a linear model of body mass against SVL, with the inclusion of second- and third-order polynomial terms (as allometry predicts a cubic relationship between length and mass). This index represents how much lighter or heavier an individual is compared to a prediction based on its length, with greater mass likely to indicate greater energy reserves and hence better condition (Schulte-Hostedde, Zinner et al. 2005).

For model selection, a full sub-model set was generated and models were ranked according to their AICc values. Models with ΔAIC < 2 relative to the lowest value were considered to be equally supported as the best models to explain the data and were averaged. "Full" (as opposed to "conditional") coefficients are quoted in the final model, meaning that any terms not appearing in a given component model were assigned a coefficient of zero before averaging.

Since previous work indicates that *B. microti* infection reduces the chance of acquiring *Bartonella* (Sherlock, Xifara et al. 2013; also see section 3.5 in this paper), we tested for indirect effects of *B. microti* on expression of selected genes, condition and fitness measures, via its effect on *Bartonella*. For models in which both *B. microti* and *Bartonella* were predictors, and *Bartonella* was found to be significant, we ran a further model in which we excluded *Bartonella* as a predictor. Our hypothesis was that, if the removal of *Bartonella* from the model caused a clear change in the size or direction of effects of *B. microti* infection, then this would be evidence of an indirect effect.

2.6.1. Spleen mass, PCV and body mass

Each of spleen mass, PCV and body mass were modelled using similar Generalised Linear Models (GLMs), with predictors including *Bartonella* and *B. microti* infection status, SVL (3rd order polynomial) and, except in the body mass model, condition. Due to the way in which we defined condition (see 2.6 above), if it were included as a predictor in the body mass model it would create a circular argument as, along with SVL, it would explain 100 % of the variation in body mass. Because of the slight change in extraction method described in 2.3, we also included interaction terms of *B. microti* and *Bartonella* with year.

2.6.2. Immunology from spleen cultures

We created a summary measure for the expression levels of genes coding for our three Th1-associated cytokines (IFNγ, Tbet and IL-2) by Principal Components analysis. The first Principal Component represented 42.6 % of the variation and correlated positively with all three gene expression variables, so we used this as our Th1 index. To test for an association between Th1 cytokines and haemoparasite infection, we ran a GLM with the Th1 index as the response variable. Predictors included presence/absence of *B. microti* and *Bartonella*, and their interactions with year (see 2.6.1 above),

2.6.3. Immunology from peripheral blood

We used the longitudinal data to investigate the immunological changes that followed infection. We constructed three separate models of the changes in expression for Gata3, IL-10 and IFNγ respectively, based on the tail blood samples. We selected all intervals for which an individual was captured in successive trapping sessions (i.e. less than 5 weeks apart) and was free from the infection in question at the start of the interval. For individuals with more than one such interval, we selected one interval at random in order to avoid pseudoreplication (we had previously attempted a mixed modelling approach, with individual as a random factor to allow the inclusion of all intervals, but the models did not converge because many individuals had only one valid interval). In order to verify that results were robust to the subsample obtained, we obtained models from 100 different random subsamples and recorded the proportion of these in which each predictor was significant (i.e. the 95 % confidence interval for the given model coefficient did not overlap with 0).

We modelled the change in gene expression over the sampling interval (natural log(x2/x1) / interval length, where x1 is the expression at the start of the interval and x2 is the expression at the end) as our response variable. Predictors included the infection status for both *B. microti* and *Bartonella* (N = uninfected, B = infected) at both the start and end of the interval. This resulted in four possibilities: NN (uninfected), NB (acute infection, acquired during the interval), BB (chronic infection, acquired previously) and BN (cleared infection, for *Bartonella* only). As *B. microti* is known to persist indefinitely (Lykins, Ristic et al. 1975, Telford, Gorenflot et al. 1993, Bown, Lambin et al. 2008), we excluded three intervals in which *B. microti* was apparently cleared (BN), on the assumption that these represented technical errors. We also included body mass as a predictor, with a quadratic term to allow for a non-linear relationship.

2.6.4. Susceptibility to infection

Using the longitudinal data, we evaluated the effect of immunology on the acquisition of infection. In two separate models, we examined the probability of an individual acquiring either *Bartonella* or *B. microti* in a given time interval. We selected time intervals as described in 2.6.3 above. We modelled the outcome of whether or not the individual became infected during the focal interval using a binomial GLM. Predictors

included level of tick and flea infestation, body mass (with a quadratic term) and expression of Gata3, IL10 and IFNγ, all measured at the start of the interval in question.

2.6.5. Mortality

We analysed mortality rates within the longitudinal samples, excluding individuals that were only captured on a single occasion, on the assumption that many of these would have been non-resident. Recapture rates were very high: a simple Capture-Mark-Recapture (CMR) model (Cormack-Jolly-Seber) with constant values for recapture and survival gave a mean recapture rate of 0.89 (95 % confidence limits 0.85 – 0.92). As a result, we judged that to calculate separate recapture and survival probabilities for each model term would greatly increase the number of model parameters for little ultimate gain in accuracy. Therefore, we decided against fitting a full CMR model and made the simplifying assumption that an individual had died if it was released and never recaptured at subsequent sampling sessions. Although we used all time intervals in order to classify survival, we excluded intervals of more than one session (> 5 weeks) from the final model, on the basis that infection status is more likely to change (undetected) during longer intervals.

We modelled survival using a Cox proportional hazards model, with predictors including *B. microti* and *Bartonella* infection status and body mass (with a quadratic term), all measured from the start of the sampling interval. Assessment of the proportional hazards assumption using Schoenfeld residuals (Schoenfeld 1982) showed that hazards for the “site” term were not constant over time. We therefore stratified by site, after which all remaining terms showed proportional hazards.

2.6.6. Fecundity

We used pregnancy rates to estimate fecundity among adult females. Again, we based our analysis on intervals between two consecutive sessions (excluding those from February and November, which are outside the breeding season), with predictors taken from the start of the interval and the outcome (pregnancy) measured at the end of the interval. We modelled pregnancy using a Cox proportional hazards model, with the inclusion of a “cluster” term which adjusts variance estimates to allow for individuals with multiple pregnancy events. Given that the vole gestation period is approximately three weeks (Ranson 1934) it is safe to assume that a female recorded as pregnant at two consecutive sampling sessions has had

two different pregnancies. Predictors included *B. microti* and *Bartonella* infection status and body mass (with a quadratic term).

2.6.7. Software

We carried out all analysis in R version 3.3.1 (R Core Team 2016), making use of the following packages: "tidyverse" for data processing and visualisation (Wickham 2016); "MuMIn" for model averaging (Barton 2016); and "survival" for Cox proportional hazards models (Therneau 2015).

3. Results

3.1. Both Bartonella and B. microti are prevalent in our sample populations

A total of 920 voles were tagged over the course of the longitudinal study, with a total of 1665 trapping instances, yielding between one and seven records per individual (mean = 1.8 captures). In addition, 345 male voles were destructively sampled forming the cross-sectional dataset. *Bartonella* infections were highly prevalent within our study animals. Of the individuals captured three or more times, 91 % were infected with *Bartonella* at some point during their capture history. In the same set of individuals, *B. microti* had a lower, but still considerable, prevalence of 37 % during the course of the study (

Table 2).

3.2. B. microti is associated with splenomegaly, and Bartonella with a reduction in body mass

There was a strong, positive association between spleen mass and *B. microti* infection, with spleens of infected animals predicted to be 0.17 g heavier (95% Confidence Interval (CI) = 0.14-0.20) than those of uninfected individuals, which had a mean mass of 0.14g (Table 3, Figure 1).

PCV was not associated with either *B. microti* or *Bartonella* infections (Table 3).

We found an association between *Bartonella* and body mass, with infected voles 1.1 g lighter (95% CI = 0.1-2.1) than uninfected voles at the same time of year and at the same site (

Table 3Table 3). *B. microti* did not feature as a predictor in the final model, even if the *Bartonella* term was excluded (Table S2).

3.3. Haemoparasitic infection is not associated with changes in the Th1 immune response

Our index measuring expression of genes coding for Th1-associated proteins was not significantly associated with either *B. microti* or *Bartonella* infections ().

3.4. An increase in IL10 expression follows Bartonella infection

Expression of IL10 increased significantly upon initial infection with *Bartonella* (0.636 log units per 30 days, 95 % CI = 0.87 – 3.36; significant in 81 % of subsamples; a change of 0.636 log units corresponds to a 1.89-fold increase; Figure 2). Expression then appeared to decline (relative to uninfected animals), both in individuals that remained infected (BB) and those that cleared the infection (BN), although the 95 % CI for both of these changes included zero (

276 Table 4. The effects of infection status on an index of expression of Th1-associated genes.

	Coefa	***LCIb***	***UCIc***
Intercept	0.163	-0.343	0.668
Babesia	0.614	-0.0981	1.33
Bartonella	0.185	-0.545	0.916
Season (cos)	-0.217	-0.677	0.243
Season (sin)	**-0.718**	-1.13	-0.305

277

278 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%

279 confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are

280 not shown – see Table S3 for full model details.

281 [b] Lower confidence interval (2.5%)

282 [c] Upper confidence interval (97.5%)

283

Table 5). *B. microti* did not feature as a predictor in the final model, even if the *Bartonella* term was excluded (Table S5). Neither Gata3 nor IFNγ expression were significantly influenced by infection status (

Table 4. The effects of infection status on an index of expression of Th1-associated genes.

	Coefa	***LCIb***	***UCIc***
Intercept	0.163	-0.343	0.668
Babesia	0.614	-0.0981	1.33
Bartonella	0.185	-0.545	0.916
Season (cos)	-0.217	-0.677	0.243
Season (sin)	**-0.718**	-1.13	-0.305

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Table S3 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

294 Table 5).

295 ### *3.5. B. microti reduces infection rates by Bartonella, but not vice versa*

296 Susceptibility to *Bartonella* was significantly lower in individuals that had a pre-existing *B. microti* infection

297 (OR (odds ratio) = 0.33, 95 % CI = 0.14 – 0.76; significant in 94 % of subsamples;

Table 6). The reverse was not true: *Bartonella* infection did not influence an individual's susceptibility to *B. microti*. Males were more susceptible than females to *B. microti* infection (OR = 4.01, 95 % CI = 1.65 – 9.78; significant in 100 % of subsamples;

301 Table 6).

302 ### *3.6. No loss of fitness detected from infections*

303 Neither *B. microti* nor *Bartonella* infections significantly influenced the estimated vole mortality rates

304 (*Bartonella* OR = 1.03, 95 % CI = 0.85 – 1.26; *B. microti* OR = 0.97, 95 % CI = 0.79 – 1.18;

305

306 Table 7, Figure 3), nor did they affect female fecundity, measured as the probability of pregnancy over time

307 (*Bartonella* OR = 0.99, 95 % CI = 0.84 – 1.16; *B. microti* OR = 0.96, 95 % CI = 0.80 – 1.15;

Table 8, Figure 4).

4. Discussion

B. microti and *Bartonella* infections appear to have little impact on the fitness of field voles, despite provoking clear physiological changes. A substantial proportion of the vole population has greatly enlarged spleens due to *B. microti* infection, and yet has similar rates of mortality and pregnancy to uninfected animals. While *Bartonella* infection is associated with a reduction in body mass, this poorer condition does not translate to any detectable loss of fitness in the two measures that we examined.

It is notable that the voles can support these infections with apparently little deleterious impact, given the associated physiological changes. Most conspicuously, we find that *B. microti*-infected field voles have highly enlarged spleens compared to uninfected voles, as has been recorded in many other host species (Lykins, Ristic et al. 1975, Cullen and Levine 1987, Watkins, Moshier et al. 1991, Telford, Gorenflot et al. 1993, Homer, Aguilar-Delfin et al. 2000). Given that splenomegaly affects *B. microti*-positive individuals almost universally (Figure 1), we infer that it is not restricted to the initial, acute phase of infection, but rather it affects the animal throughout the full course of infection, including when the vole has carrier status. An enlarged spleen indicates proliferation of lymphocytes, and/or that large numbers of RBCs are being recycled from the blood (Eichner 1979, Jiao, Okumiya et al. 2001), presumably to remove the pathogen-containing cells. Despite this, we find no evidence of anaemia associated with *B. microti*, meaning it is likely that new RBCs are also being produced at an elevated rate to make up for those being removed. A study in another vole species, *M. montanus*, found a similar pattern of splenomegaly without anaemia in *B. microti*-infected individuals, and also showed that erythrocytes were on average younger in these individuals (Watkins, Moshier et al. 1991). We would expect such energetic expenditure to have a negative impact on the animal's condition or fitness, and yet, despite substantial sample sizes, we have not found evidence for any such impact.

We did not find any increase in expression of genes coding for Th1-related cytokines such as IFNγ associated with either of the focal infections. One might predict stimulation of the Th1 pathway in response to infections such as these, in accordance with its function in targeting intracellular parasites (Abbas,

Murphy et al. 1996). Indeed, in the laboratory, *B. microti* has been shown to stimulate increased expression of IFNγ and IL-2 in mice during a period one to three weeks post-infection (Chen, Copeman et al. 2000). Similarly, mice experimentally infected with *Bartonella henselae* show an increase in secretion of IFNγ indicative of a Th1 response (Arvand, Ignatius et al. 2001, Kabeya, Yamasaki et al. 2007), and in cats, a stronger Th1 response leads to reduction in *B. henselae* bacteraemia (Kabeya, Umehara et al. 2009).

By contrast, we did observe an increase in expression of IL-10 following initial infection with *Bartonella*. IL-10 is a regulatory cytokine that reduces inflammatory responses, and in other host-pathogen systems it has been shown to play a role in tissue damage associated with inflammation (Hunter, Ellis-Neyes et al. 1997, Brown, Zachary et al. 1999, Sanni, Jarra et al. 2004). Kabeya et al. (2007) observed an increase in IL-10 secretion following *B. henselae* infection, which acted to reduce the strength of Th1 response. The induction of IL-10 production by *Bartonella* may therefore help the parasite to persist in its host (Kabeya, Yamasaki et al. 2007), but, given the lack of detectable fitness cost in our study, it is also possible that IL-10 might minimise negative impacts for host health by reducing inflammation. The addition of data on parasite loads would allow more detailed investigation of this possibility.

Our study provides independent corroboration of the finding of Sherlock, Xifara et al. (2013), that *B. microti* protects against *Bartonella* infection, but that the reverse is not true. So far we have been unable to establish a mechanism for the inhibition. One possibility is that *B. microti* might cause changes in immunology that then make the individual better able to resist initial infections from *Bartonella*. Immunological changes are a common way for concurrent infections to interact (Cox 2001). For example, increased production of cytokines such as Tumour Necrosis Factor has been credited for mediating protection against protozoan diseases such as malaria and *B. microti* after infection with tuberculosis (Clark 2001). However, on the basis of the genes examined in this study, we found no evidence that *B. microti* induces changes in immune status, nor that immune status influences *Bartonella* acquisition. We note, however, that levels of gene expression do not necessarily correlate directly with concentrations of their protein products (Vogel and Marcotte 2012), and therefore direct data on circulating cytokines would allow us to investigate these immunological interactions more thoroughly.

Alternatively, the interaction between *B. microti* and *Bartonella* might be physiological. The most conspicuous feature of *B. microti* infection is splenomegaly, and this is associated with an increased number of reticulocytes in the blood compared to mature erythrocytes (Watkins, Moshier et al. 1991). There is some evidence that both *B. microti* (Borggraefe, Yuan et al. 2006) and *Bartonella* (Scheff, Scheff et al. 1956) are less likely to infect reticulocytes than mature erythrocytes. Therefore, by maintaining high reticulocyte populations, *B. microti*-infected individuals may limit the capacity of *Bartonella* and *B. microti* to reproduce, hence lowering the probability of the infections establishing/re-establishing.

In cases of negative interactions between two different parasites, we might predict an indirect, positive effect of one infection on the host via a reduction in abundance or prevalence of the other. For example, Wuerthner, Hua et al. (2017) found that a trematode infection in amphibians can have a positive fitness effect by reducing ranaviral loads. However we found no evidence of a similar positive effect of *B. microti* in the current study. For example, although we found that *Bartonella* causes a reduction in body mass, this did not translate into a positive effect of *B. microti* on mass on exclusion of *Bartonella* from the model. This may simply be because the impacts of *Bartonella* on an individual's health are relatively small, and therefore any indirect effects conferred are too small to be detected.

While we find little evidence for negative health consequences of these infections in the voles, it is plausible that our sampling method overlooked the most acutely infected individuals. In theory, an individual that was recorded as free of infection could subsequently become infected and die before recapture. In this case, the infection would not be observed, making it impossible to ascribe that death to the parasite. Given the high prevalence of among the sampled population, we can be confident that our conclusions apply to a sizable number of infected individuals, but we cannot rule out that some individuals are more acutely affected. It is also possible that we missed some fitness consequences of infection (e.g. effects on number of offspring) by focusing on mortality and pregnancy rates.

Overall, our study shows that *B. microti* and *Bartonella* have little negative impact on their field vole hosts, despite provoking clear immunological and physiological changes that, at least in the case of *B. microti*, appear to persist for long periods of time. This demonstrates the importance of using direct measures of

fitness components wherever possible to assess the impact of parasites in natural populations, rather than relying on assumptions based on pathology or prevalence of infection.

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Figure legends

Figure 1. Variation in spleen weight with body length and *B. microti* infection status. Spleen weight has been calculated as partial residuals from the model given in

527 Table 3

Table 6. Susceptibility to *B. microti* and *Bartonella*: estimated log odds ratios from binomial GLM.

	Bartonella model			***B. microti model***		
	Coefa	*LCIb*	*UCIc*	*Coef*	*LCI*	*UCI*
Intercept	0.88	-0.71	2.48	-2.56	-3.73	-1.38
B. microti (infected)	**-1.12**	-1.96	-0.27			
Bartonella (infected)				-0.06	-0.51	0.39
Sex (M)	0.1	-0.43	0.62	**1.39**	0.5	2.28
Body mass				3.24	-1.69	8.16
Body mass2				**7.6**	2.52	12.7

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. The binomial model returns log odds ratios, so odds ratio = ecoef. Some terms with confidence intervals that overlap zero are not shown – see Table S6 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

. The effects of *Bartonella*, breeding status and site have been standardised to their modal values, and condition and season to their median values. Points show partial residuals for each individual (solid = infected with *B. microti*, outline = uninfected), lines show model predictions (solid = infected, dotted = uninfected) ± standard error in grey.

Figure 2. Fold changes in IL-10 expression according to *Bartonella* infection status. Changes are standardised for a typical sampling interval (30 days) and plotted on a log scale. Values have been converted to partial residuals using the model given in Table 5. The effects of *B. microti*, sex, breeding status and site have been standardised to their modal values, and weight change and season to their median values. Boxes show the inter-quartile range (IQR) with a thick horizontal line for the median.

Whiskers extend to the smallest/largest values that are within 1.5 x IQR of the box, and values beyond that are shown as individual points.

Figure 3. Survival of voles according to infection status. Lines show the proportion of individuals estimated from capture records to have survived at different time points. Solid line = neither infection (93 records, 34 events), dashed line = *B. microti*-infected (97 records, 36 events), dotted line = *Bartonella*-infected (278 records, 117 events), dot/dash line = both infections (52 records, 23 events). Note that the reported 100% survival rate over the first 50 days is an artefact due to our exclusion of individuals with only a single capture record.

Figure 4. Female fecundity according to infection status. Lines show the proportion of individuals with at least one recorded pregnancy at different time points. Solid line = neither infection (73 records, 44 events), dashed line = *B. microti*-infected (33 records, 20 events), dotted line = *Bartonella*-infected (156 records, 83 events), dot/dash line = both infections (15 records, 7 events).

Tables

Table 1. Summary information of model specifications, including predictors from each full model before simplification.

Predictors	*Th1 index*	*Spleen mass*	*PCV*	*Body mass*	*Change in Gata3*	*Change in IL10*	*Change in IFNγ*	*Susceptibility to Bartonella*	*Susceptibility to B. microti*	*Mortality*	*Fecundity*
site	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
season	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
sex					Y	Y	Y	Y	Y	Y	
mating status	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
SVL (3rd order polynomial)		Y	Y	Y							

body mass (2nd order polynomial)								Y	Y	Y	Y
body mass change					Y	Y	Y				
condition		Y	Y								
B. microti	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y
Bartonella	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y
ticks								Y	Y		
fleas								Y	Y		
IL10								Y	Y		
Gata3								Y	Y		
IFNγ								Y	Y		
year:B. microti	Y	Y	Y	Y							
year:Bartonella	Y	Y	Y	Y							
dataset[a]	C	C	C	C	L	L	L	L	L	L	L
n	142	285	278	286	192	181	191	121	190	296	127
Degrees of freedom	11	15	15	14	10	10	10	14	14	11	9

[a] **C = cross-sectional. L = longitudinal.**

566 **Table 2. Prevalence of selected parasites within the sampled voles.**

Parasite	*Point prevalence*[a]	*"Lifetime" prevalence*[b]
Bartonella	0.57	0.91
B. microti	0.18	0.37
Flea	0.54	0.92
Tick	0.21	0.57

567

568 [a] The probability that, at any given capture, an individual will be found to be infected with the parasite.

569 [b] The proportion of individuals with three or more captures in which the parasite has been detected on at least one

570 occasion.

571

Table 3. The effects of infection status and body size on three physiological parameters: spleen mass, packed cell volume, and body mass, estimated from GLMs.

	Spleen mass (g) model			*Packed Cell Volume (%) model*			*Body mass (g) model*		
	Coef[a]	*LCI*[b]	*UCI*[c]	*Coef*	*LCI*	*UCI*	*Coef*	*LCI*	*UCI*
Intercept	**0.186**	0.134	0.238	**51.2**	49.7	52.7	**24.8**	23.5	26.2
SVL	**1.738**	1.314	2.162	-9.2	-22.9	4.5	**110.5**	99.6	121.4
SVL²	**0.242**	0.027	0.456	**-14.3**	-24.8	-3.8	**10.8**	3.8	17.7
SVL³	**-0.271**	-0.472	-0.071				**-16.8**	-23.4	-10.2
Mass residuals	**0.012**	0.009	0.016						
B. microti (infected)	**0.172**	0.143	0.201						
Bartonella (infected)							**-1.1**	-2.1	-0.1
Status (breeding)							**2.3**	0.9	3.7
Season (cos)							**-3.2**	-4	-2.3
Season (sin)							**0.6**	0	1.3
Site (SQC)				0.1	-1.4	1.6	-0.8	-1.8	0.1
Site (KTH)				**-2.8**	-4.9	-0.7	**2.2**	0.8	3.6
Site (SCP)				**-2.8**	-5	-0.5	**1.6**	0.2	3.1

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Table S1 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

583 **Table 4. The effects of infection status on an index of expression of Th1-associated genes.**

	Coef[a]	*LCI*[b]	*UCI*[c]
Intercept	0.163	-0.343	0.668
Babesia	0.614	-0.0981	1.33
Bartonella	0.185	-0.545	0.916
Season (cos)	-0.217	-0.677	0.243
Season (sin)	**-0.718**	-1.13	-0.305

584

585 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%
586 confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are
587 not shown – see Table S3 for full model details.

588 [b] Lower confidence interval (2.5%)

589 [c] Upper confidence interval (97.5%)

590

Table 5. Change in gene expression: log fold change per 30 days, estimated from GLMs.

	IL10 model			*Gata3 model*			*IFNγ model*		
	Coef[a]	*LCI*[b]	*UCI*[c]	*Coef*	*LCI*	*UCI*	*Coef*	*LCI*	*UCI*
Intercept	0.36	-0.123	0.846	0.063	-0.081	0.21	0.072	-0.015	0.159
B. microti (NB)	0.261	-0.174	0.699						
B. microti (BB)	-0.153	-0.468	0.165						
Bartonella (NB)	**0.636**	0.261	1.008						
Bartonella (BB)	-0.204	-0.537	0.129						
Bartonella (BN)	-0.345	-0.741	0.048						
Status (breeding)				**-0.105**	-0.192	-0.015			

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Table S4 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

Table 6. Susceptibility to *B. microti* and *Bartonella*: estimated log odds ratios from binomial GLM.

	***Bartonella* model**			***B. microti* model**		
	Coef[a]	*LCI*[b]	*UCI*[c]	*Coef*	*LCI*	*UCI*
Intercept	0.88	-0.71	2.48	-2.56	-3.73	-1.38
B. microti (infected)	**-1.12**	-1.96	-0.27			
Bartonella (infected)				-0.06	-0.51	0.39
Sex (M)	0.1	-0.43	0.62	**1.39**	0.5	2.28
Body mass				3.24	-1.69	8.16
Body mass^2				**7.6**	2.52	12.7

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. The binomial model returns log odds ratios, so odds ratio = e^{coef}. Some terms with confidence intervals that overlap zero are not shown – see Table S6 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

611 **Table 7. Cox proportional hazards model of individual mortality.**

	Coef[a]	*LCI*[b]	*UCI*[c]
Bartonella (infected)	0.037	-0.160	0.233
B. microti (infected)	-0.034	-0.233	0.165
Sex (M)	**0.526**	0.206	0.846
Status (breeding)	**0.445**	0.044	0.845
Body mass	**-23.1**	-28.8	-17.5
Body mass^2	**4.68**	0.69	8.68
Season (cos)	-0.133	-0.887	0.621
Season (sin)	**1.37**	0.99	1.76

612 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%
613 confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are
614 not shown – see Table S7 for full model details.

615 [b] Lower confidence interval (2.5%)

616 [c] Upper confidence interval (97.5%)

617

618

Table 8. Cox proportional hazards model of female fecundity.

	Coef[a]	*LCI*[b]	*UCI*[c]
B. microti (infected)	-0.01	-0.18	0.15
Bartonella (infected)	-0.04	-0.22	0.14
Body mass	**-3.97**	-7.58	-0.35
Body mass^2	**-3.84**	-6.76	-0.91
Season (cos)	**-0.64**	-1.09	-0.19
Season (sin)	0.09	-0.24	0.43

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Table S8 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)